EXHIBIT 2

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Ex vivo response to aspirin differs in stroke patients with single or recurrent events: a pilot study

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Abstract

The dose of aspirin for secondary stroke prevention and the clinical meaning of ex vivo platelet abnormalities are debated. We assessed prospectively 39 noncardioembolic stroke patients in which 300 mg/day aspirin had proved effective (n=24) or ineffective (n=15) to prevent recurrent ischemic events. We evaluated platelet aggregation induced by arachidonic acid, adenosine diphosphate and epinephrine, and the sensitivity of platelets to increasing concentrations of the synthetic thromboxane mimetic U46619. Aggregation studies were repeated while subjects received 300 (study phase 1), and 600 (study phase 2) mg/day aspirin, respectively. Overall, arachidonic acid-induced platelet aggregation was less effectively inhibited during study phase 1 compared to phase 2. Arachidonic acid and epinephrine promoted a stronger platelet aggregation in aspirin nonresponders than in aspirin responders while taking 300 mg/day aspirin. On the other hand, 600 mg/day effectively inhibited platelet function in both clinical groups. A lower sensitivity to thromboxane receptors was also found during phase 1 of the study, although the response was similar between aspirin responders and nonresponders. This pilot study suggests that 300 mg/day aspirin is less effective than 600 mg/day to block the cyclooxygenase pathway in noncardioembolic stroke and, incomplete cyclooxygenase inhibition is associated with recurrent thromboembolic events despite adequate aspirin compliance. It is likely that patients could receive a more efficacious stroke prevention if the dose of aspirin is tailored to individual needs as reflected by laboratory findings.

Keywords: Cerebrovascular disease; Aspirin; Platelets

1. Introduction

Randomized clinical trials and meta-analyses have reported a modest but significant effect of aspirin for secondary stroke prevention in patients with prior myocardial infarction, acute myocardial infarction, prior stroke, or TIA [1,2]. It is estimated that 30–300 mg aspirin daily prevents only 13% (95% CI: 4–21%) of the vascular complications in patients who had an episode of cerebral ischemia [3]. As equivalent efficacy rates were found for low-, medium- and high-dose aspirin, the Antiplatelet Trialists' Collaboration [1] concluded that '... there appears to be no good reason to use a dose higher than 300–325 mg/day ...' Regardless of the large number of patients included in these analyses this recommendation has not received unanimous acceptance [4–6]. Likewise, leading stroke experts disclose substantial dose differences when prescribing aspirin for secondary stroke prevention [7,8]. A few studies [9–15] have looked for biological markers which might help to select the most appropriate dose of aspirin in stroke patients. However, lacking adequate correlation between clinical and laboratory findings, these studies did not resolve the clinical significance of ex vivo response of platelets to aggregation stimuli.

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Subsequently, it is not known whether a variation in aspirin dose is more suited than a change of therapy for secondary prevention in stroke patients with recurrent events despite adequate aspirin compliance. To provide further light on this controversy we assessed a cohort of noncardioembolic stroke patients in which 300 mg/day aspirin had proved effective (aspirin responders) or ineffective (aspirin nonresponders) to prevent recurrent events. The ex vivo response of platelets exposed to several agonists was compared between aspirin responders and nonresponders while receiving increasing doses of aspirin. Based on the results obtained it is argued that stroke patients could receive a more efficacious antiplatelet therapy if the dose of aspirin is tailored to individual needs as reflected by laboratory findings.

2. Methods

From October 1992 to May 1995, we admitted 54 patients into our Stroke Unit with recurrent stroke within less than 12 months and for whom a dose of 300 mg/day of enteric-coated aspirin had been first prescribed. Based on clinical grounds we classified these patients as 'aspirin nonresponders'. A contemporary group of 25 first-ever stroke patients followed at our Stroke Clinic was classified as 'aspirin responders' as 300 mg/day of enteric-coated aspirin had successfully prevented further recurrent events during a follow-up period of at least 24 months. Both groups of patients were matched for age, sex and stroke type. All of them had a brain-CT scan or a brain-MR to document the ischemic nature of the infarction. Appropriate diagnostic tests, including electrocardiography, echocardiography, Doppler ultrasounds, cerebral angiography or magnetic resonance angiography were performed in all instances to exclude a cardiac source of emboli. The prevalence of atherosclerotic risk factors, the severity of neurological impairment (Mathew score scale), and the size of the infarction were also recorded using previously described methods [16]. Aspirin compliance was assessed by interviews with the patients or their relatives if there was cognitive impairment. To avoid the effects of confounding factors on stroke incidence, patients were excluded from further study if any of the following: cardioembolic stroke ($n=20$), admittance of irregular aspirin compliance ($n=13$), or lack of written consent ($n=7$). The remainder 39 patients were included in the prospective phase of the study.

The prospective part of the study was initiated at least 6 months apart from the last clinical event to avoid the confounding effect of acute stroke on normal platelet function. Then, aspirin responders and nonresponders were given 300 mg/day nonenteric-coated aspirin for 7 days (study phase 1), and then 600 mg/day nonenteric-coated aspirin for 7 additional days (study phase 2). The 2-weeks medication was provided by a neurologist who encouraged the patients to comply with the prescribed medication and who assessed compliance at the end of phase 2 by simple pill counting. None of the patients experienced recurrent events or untoward side-effects during phase 1 or 2 of the study.

Platelet aggregation studies were performed at day 7 of phase 1 and at day 7 of phase 2, respectively, by a hematologist blind to aspirin dose and clinical data. Blood samples were obtained by venipuncture and rapidly mixed with citrate–phosphate–dextrose. The final concentration of citrate in the samples was 19 mM. Platelet rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by differential centrifugation. Platelet aggregation was measured according to the method of Born using a Hitachi Aggrecorder (Kyoto Dai-Ichi Kagaku Co. Ltd, Kyoto, Japan) [17]. The ability of PRP to aggregate was tested with 1.4 μmol/l arachidonic acid (AA), 1–2 μmol/l adenosine diphosphate (ADP) and 10 μmol/l epinephrine (EPI). All aggregating agents were obtained from Menarini Diagnostics, Barcelona, Spain. The sensitivity of platelets to respond to the synthetic thromboxane mimetic U46619 (Upjohn Co., Kalamazoo, MI) was also tested in a range of concentrations from 0.3 to 1.2 μmol/l. Results of aggregation studies were always expressed as percentage of maximal aggregation as previously described [17].

3. Statistical analysis

Continuous data were reported as mean±standard deviation (S.D.) and non-continuous data as number (%). Factorial and repeated measures ANOVA were used for continuous variables and chi-square for dichotomous variables. Sensitivity to U46619 was plotted as proportion of samples >40% of maximal aggregation±S.E. of proportions. Differences between 300 and 600 mg aspirin were assessed by comparison of proportions for each concentration of U46619. A P value <0.05 was set as statistically significant. The BMDP statistical software was used for analysis [18].

4. Results

4.1. Characteristics of the study population

Thirty-nine patients were finally entered in the study, including 14 aspirin nonresponders and 25 aspirin responders. Main characteristics of both groups are shown in Table 1. With the exception of a greater prevalence of diabetes mellitus among aspirin nonresponders no other significant differences in vascular risk factors were observed between the two aspirin groups.

Table 1
Main characteristics of the study cohort

	Responders (n=25)	Nonresponders (n=14)
Age (years)	65.2 (9.0)	69.7 (6.1)
Male/female	76/24	79/21
Hypertension	15 (60)	4 (29)
Diabetes*	3 (12)	6 (43)
Ischemic heart disease	4 (16)	3 (21)
High cholesterol	4 (16)	6 (43)
Infarct volume (cc)	18.0 (35.7)	16.2 (32.6)
Large vs. small vessel stroke	14/11	7/7
Mathew score	89.1 (12.9)	93.2 (8.1)

* P<0.05; data are means (S.D.) or numbers (%).

4.2. Inhibition of platelet aggregation in relation to aspirin dose (300 or 600 mg/day)

As shown in Table 2, AA-induced platelet aggregation was less effectively inhibited in the total cohort while patients were taken 300 mg/day aspirin. ADP and EPI induced similar platelet aggregation while patients took 300 or 600 mg/day aspirin.

4.3. Inhibition of platelet aggregation in relation to clinical efficacy (aspirin responders and nonresponders)

Further analyses disclosed in Table 3 show incomplete inhibition of AA- and EPI-induced platelet aggregation only in aspirin nonresponders. Moreover, complete inhibition of platelet aggregation was achieved when the daily dose of aspirin was increased to 600 mg. On the other hand, aspirin responders disclosed normal platelet inhibition following aggregation stimuli irrespective the daily dose of aspirin.

Table 2
Platelet aggregation studies in relation to aspirin dose in 39 patients*

	Aspirin	
	Phase 1 300 mg/day	Phase 2 600 mg/day
AA (1.4 μmol/l)†	41.1±39.4	17.2±15.7
ADP (1 μmol/l)	58.4±23.9	52.7±17.9
ADP (2 μmol/l)	80.1±17.7	77.3±20.3
EPI (10 μmol/l)	67.6±24.9	59.2±21.5

* Data are mean (S.D.).
† P<0.001.

Table 3
Platelet aggregation in aspirin responders and nonresponders*

Aspirin dose (mg)	Nonresponders (n=14)	Responders (n=25)
AA (1.4 μmol/l)***		
300	70.8±39.1**	24.42±28.7
600	15.2±11.5	18.2±17.5
EPI (10 μmol/l)†		
300	82.3±23.2*	59.4±22.2
600	62.4±18.0	57.6±23.2

* Data are mean (S.D.).
* P<0.01 (responders versus nonresponders); ** P<0.0001 (responders versus nonresponders); *** P<0.001 (300 versus 600 mg/day); † P<0.01 (300 versus 600 mg/day).

4.4. Sensitivity to thromboxane agonist U46619

A statistically significant lower sensitivity to thromboxane agonist U46619 was also found during phase 1 of the study. However, the sensitivity to U46619 did not differ between aspirin responders and nonresponders during phase 1 (83.4±15.5 vs. 84.6±11.3) or phase 2 (90.0±7.5 vs. 89.6±4.7), respectively.

5. Discussion

Previous experimental studies have suggested that the effects of aspirin on platelet function are dose-dependent as higher doses were required to achieve complete platelet inhibition [9–12,23]. In agreement with these findings we also observed that medium-dose aspirin (300 mg/day) was less effective than high-dose aspirin (600 mg/day) to offset platelet aggregation induced by AA in a very well-defined population of patients with noncardioembolic stroke. However, unlike previous larger studies in which the link between laboratory abnormalities and clinical outcome was not established, the major new finding of the study was that the inhibitory effect of 300 mg/day aspirin was particularly weak in patients in which this dose had proved on clinical grounds to be incompetent to prevent further ischemic events (aspirin nonresponders). Although it could be argued that aspirin failure merely reflects a more advanced form of atherosclerosis in these patients we also believe that the dose of aspirin could be related with its lack of clinical efficacy. In support of this belief is the fact that aspirin responders and nonresponders shared most of the clinical and epidemiological attributes that determine the severity of atherosclerosis and that the diagnostic work-up did not show major differences between the aspirin groups that were defined. Moreover, aspirin nonresponders disclosed while taking 300 mg/day aspirin an incomplete inhibition of AA- and EPI-induced platelet aggregation that was overcome when the dose of aspirin was increased to 600 mg/day.

In apparent contention with these findings we also observed a statistically significant lower sensitivity to the thromboxane mimetic U46619 while patients were receiving the smaller dose of aspirin (study phase 1). However, we could not find a relationship between the sensitivity to stable analogues of thromboxane A_2 and the clinical response to aspirin, as responders and nonresponders disclosed similar values. For aspirin is a relatively selective inhibitor of the constitutive isoform COX-1 it is more likely that the insufficient antiplatelet effect of aspirin resulted from the induction of aspirin-resistant COX-2, or

from a different sensitivity of hyperreactive platelets against aspirin subsequent to activation by thromboxane-independent pathways [19]. Unfortunately, as patients were not followed clinically after the aggregation studies were performed we cannot forecast whether aspirin nonresponders would have benefited clinically from an increase in aspirin dose. However, this prediction is supported by the fact that complete platelet inhibition was achieved once the dose of aspirin was increased.

The Antiplatelet Trialists' Collaboration showed equivalent efficacy rates for high- and low-dose antiplatelet agents [1]. However, this widely quoted meta-analysis has been criticized for evaluating dissimilar groups of clinical trials, heterogeneous clinical end-points and mixed antiplatelet agents [4]. A recent mini-metaanalysis restricted to the efficacy of various doses of aspirin in stroke patients only concluded that aspirin at any dose above 30 mg daily prevented a modest 13% (95% CI: 4–21%) of vascular events [3].

The value of ASA for stroke prevention has also been evaluated in several surgical trials. A post hoc analysis of the perioperative stroke risk in NASCET detected a five-fold increase in stroke for patients taking 325 mg/day aspirin or less compared with those taking 650 mg/day or more [20]. More recently, the ASA and Carotid Endarterectomy (ACE) trial reported that the risk of stroke, myocardial infarction, and death within 30 days and 3 months of endarterectomy was lower for patients taking 81 mg or 325 mg ASA daily than those taking 650 mg or 1300 mg [21]. Although the trial supported the use of a low-dose acetylsalicylic acid in patients scheduled for carotid endarterectomy the investigators suggested the need for caution in the extension of these results to the long-term management of non-surgical patients. In the accompanying commentary to this study [22], the editorialist concluded that the opposing observation encountered in the NASCET and ACE trials may well represent another fluctuation around essentially the same antithrombotic effect for any dose of aspirin between 30 mg and 1300 mg. However, we ignore whether the incidence of treatment failures observed in both surgical trials would have decreased if the dose of ASA given to patients had been adjusted to individual needs as reflected by platelet functional assays.

The small number of patients included in the pilot study calls for a note of caution in its interpretation. However, the study evaluated prospectively a very homogeneous series of stroke patients whose main distinction was the clinical response to the same dose of aspirin. Therefore, our data contradict the view that similar clinical effects are expected in all stroke patients irrespective the dose of aspirin. Nevertheless, as platelet response to aspirin may be variable even in normal subjects [23] direct comparisons of different aspirin doses will be needed to terminate the aspirin wars. Unlike the NASCET and ACE trials these comparisons should not be restricted to surgical patients but extended to all patients with vascular disease. Meanwhile, our preliminary results suggest that targeting the dose of aspirin to individual patient's needs as reflected by means of ex-vivo platelet function assays might increase the net clinical efficacy of aspirin for stroke prevention.

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